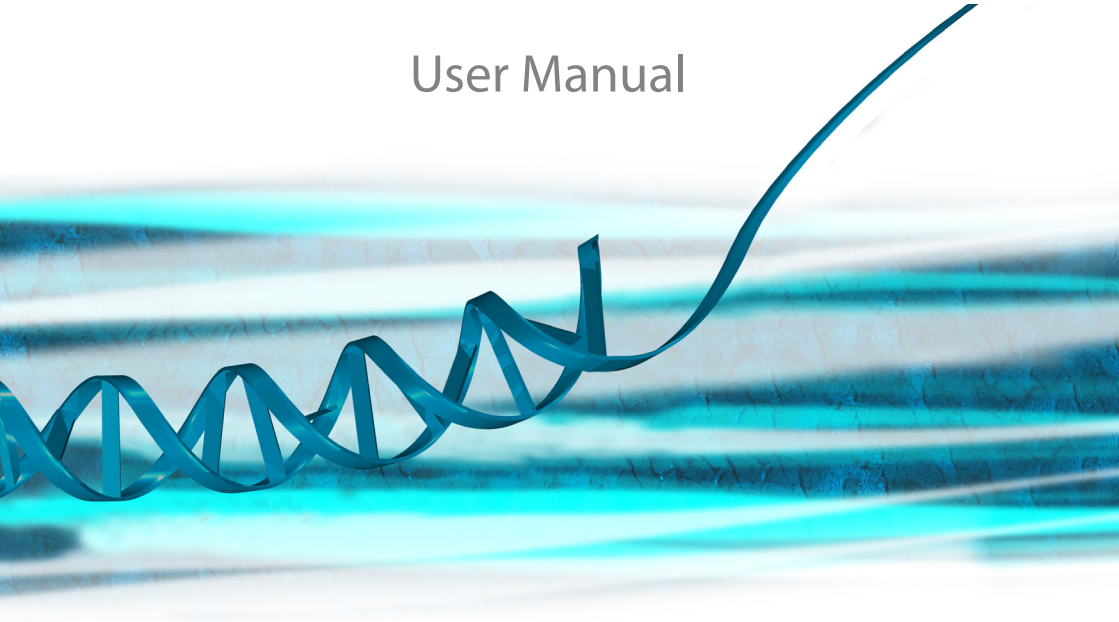
User Manual



TATAA Interplate Calibrator

Probe protocol (FAM or VIC/JOE)

250/1000 rxn

Version 1.1 — August 2012

For use in quantitative real-time PCR

tataabiocenter

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Background

For practical reasons many qPCR studies involve the use of samples that are processed in more than a single batch or in which the sample set is extended over time. Even over a short time period, variation between qPCR processing runs is observed due to different baseline subtractions and threshold settings. The bias is NOT introduced when using the "all samples" or "all assays" plate layout and performing $\Delta\Delta C_q$ based analysis, but it is the "mixed" layout for which interplate calibration is needed (Figure 1). The TATAA Interplate Calibrator (IPC) is used to compensate for the variation between qPCR runs. The TATAA IPC sample material is provided in ready-to-use aliquots and is a very stable template that is amplified with a highly robust assay. The TATAA IPC should be included in all qPCR runs. Any differences in the measured IPC C_q values among the runs reflect the bias introduced by the instrument and is compensated for.

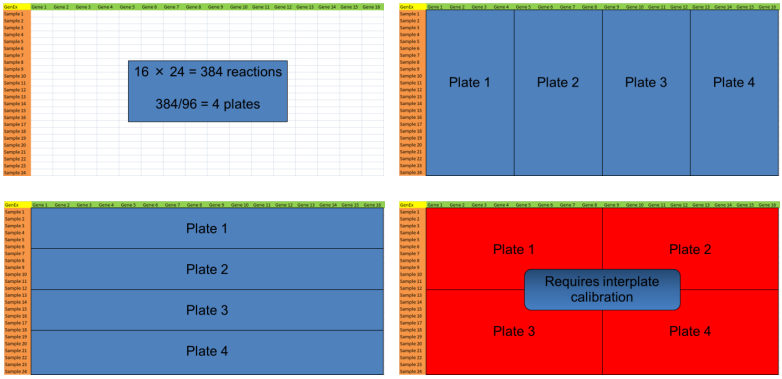


Figure 1: Different options for the design of a multi-plate qPCR study for $\Delta\Delta C_q$ based analysis. Top left: the study contains 24 samples and 16 genes and requires four runs with 96-well block instruments (y-axis: samples, x-axis: genes). Top right: "All samples" are always assayed on the same plate. Bottom left: "All genes" are always assayed on the same plate. Bottom right: "Mixed" design which requires interplate calibration.

The TATAA IPC is also suited for *absolute quantification*. The recommended strategy is to construct a single, highly precise standard curve for your target gene. Base it on large number of standards covering a wide concentration range

(recommended 7-9 concentrations, each run in 3-4 replicates). This standard curve is then used for interpolation of all field samples. The field samples may be measured over time in independent runs. Proper interpolation then requires that the IPC sample is included in each run. This approach is much more accurate than running a separate standard curve in each run, particularly if based on a smaller number of standards, since the random noise in the separate standard curves introduces systematic run-to-run variation (Tichopad 2012).

The C_q of the IPC is measured with high accuracy. The TATAA IPC has been extensively optimized to reliably and predictably amplify, providing highly reproducible C_q values. However, it is still recommended as good practice to run technical replicates of the TATAA IPC (preferably at least triplicates, Figure 2). Poor assays should never be used as interplate calibrators, since the noise contributed by these measurements may in turn worsen the quality of the data rather than improving it. It is sufficient to run one set of IPC replicates for each instrument channel used within a study if a common threshold is set. Hence, for most singleplex assay, technical replicates of a single IPC are sufficient. It is not recommended to perform separate inter plate calibrations for each assay, since the noise contributed by the independent corrections is likely to reduce data quality.

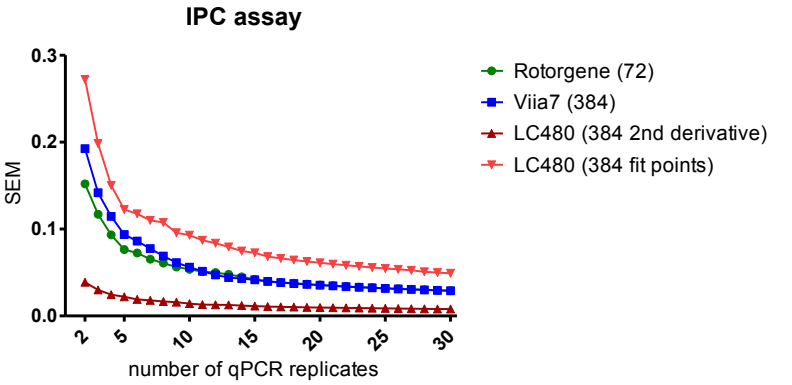


Figure 2: The relationship between the standard error of the mean (SEM) and the number of replicates used in runs with identical settings on different instruments. LC480 shows two different threshold settings on one run.

TATAA Interplate calibrator is:

- provided in aliquots (stored at -20°C) for easy and flexible use and long term stability.
- a robust assay that performs excellent in most master mixes and over a wide range of annealing temperatures.
- a stable template at optimum concentration that produces $C_q \approx 15$ -20 under most conditions.

Contents

- Interplate Calibrator (IPC) template:
  50 aliquots @ 20 µl (c = 10^6 copies / µl)
  or 200 aliquots @ 20 µl (c = 10^6 copies / µl)

- IPC primers:
  5 aliquots @ 50 rxns* (250 µl of primer mix, c = 2 µM per primer)
  or 20 aliquots @ 50 rxns* (1000 µl of primer mix, c = 2 µM per primer)
- IPC probe:
  5 aliquots @ 50 rxns* (125 µl of solution, c = 2 µM per primer)
  or 20 aliquots @ 50 rxns* (500 µl of solution, c = 2 µM per primer)

*rxns = qPCR reaction in 25 µl, concentration = 400nM per primer, 200 nM probe

The IPC assay produces a 100 bp amplicon with very high PCR efficiency (E > 90% in tested commercial master mixes) and produces negligible primer dimer products. The probe assay is available in two variants, either with FAM or with CalFluorGold540 (equivalent to VIC, JOE) reporter, both with BHQ1 quencher.

Additionally required materials and devices

• Real-time PCR instrumentation

The TATAA Interplate Calibrator has been validated on the Roche LightCycler 480, Biorad CFX 96/384, Stratagene MxPro, Rotorgene, ABI 7500 Fast, Eppendorf Realplex, Illumina Eco, Fluidigm BioMark and is expected to perform well on equivalent instruments. The probe signal shall be measured on the instrument's FAM or VIC (JOE) channel, depending on the probe included.

• Master mix

The TATAA Interplate Calibrator assay has been validated in a large number of mastermixes using conditions recommended by the manufacturers (Table 1):

Master mix	Final concentrations*	Annealing temperature
Applied Biosystems TaqMan® Gene expression Master Mix	800 nM primer, 200 nM probe	= 60°C (59-65)
Applied Biosystems TaqMan® Universal PCR Master Mix	800 nM primer, 200 nM probe	= 60°C (60-65)
Applied Biosystems TaqMan® Genotyping Master Mix	800 nM primer, 200 nM probe	= 63°C (63-65)
Finnzymes DyNAmo™ Flash Probe qPCR Kit	500 nM primer, 250 nM probe	= 60 °C (59-63)
Finnzymes DyNAmo™ ColorFlash Probe qPCR Kit	500 nM primer, 250 nM probe	= 60 °C (59-63)
KAPA™ PROBE FAST qPCR Kit	400 nM primer, 200 nM probe	= 60 °C (57-64)
PrimerDesign Precision qPCR MasterMix	300 nM primer, 150 nM probe	= 60 °C (59-64)
Qiagen QuantiTect Probe PCR Kit	400 nM primer, 200 nM probe	= 60 °C (59-65)
Quanta PerfeCTa® qPCR FastMix® II	500 nM primer, 250 nM probe	= 60 °C (55-65)
Roche LC480 Probes master	500 nM primer, 200 nM probe	= 60 °C (55-65)
TAKARA Premix Ex Taq (probe qPCR)	200 nM primer, 200 nM probe	= 60 °C (55-65)
TATAA Probe GrandMaster® Mix	500 nM primer, 250 nM probe	= 60 °C (55-65)

* Concentration of each primer per qPCR

Table 1: Recommended primer and probe concentrations and annealing temperatures in selected commercial master mixes. Acceptable ranges of annealing temperatures to synchronize TATAA Interplate calibrator assay with other experimental assays are shown within parenthesis. TATAA IPC assay performance has been validated within these temperature ranges.

• Pipettes and tips (available from www.tataa.com)

• Vortex and centrifuge

• Experimental sample DNA/cDNA

• Optionally reference cDNA and gDNA

New assays can be validated on cDNA and DNA libraries available from TATAA (www.tataa.com) for mouse, human or rat.

Storage

For long term storage, store the TATAA Interplate Calibrator aliquots at -20°C. The TATAA IPC is stored in stabilizing buffer and shows no degradation within a week at room temperature or after four freeze-thaw cycles (Figures 3, 4). The primer mix and probe may be stored at -20°C for up to a year or at +4°C for up to one month. Avoid repeated freeze-thaw cycles, use the provided aliquots instead.

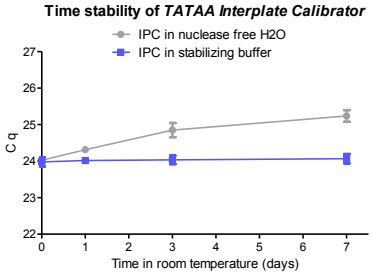


Figure 3: Stability of TATAA Interplate calibrator template in time in room temperature.

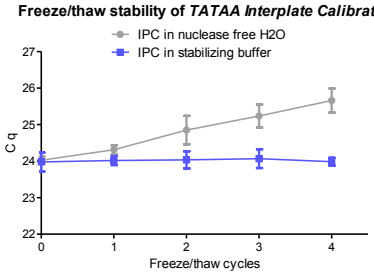


Figure 4: Stability of TATAA Interplate calibrator template after repeated cycles of freezing in -80 °C and thawing.

Interplate calibration - additional information

A basic requirement for a simple and efficient interplate calibration is having parallel amplification curves of all the assays in all the samples compared in the experiment (Figure 3). To achieve that, all assays should be validated, and any inhibited reaction should be excluded (MIQE guidelines, Bustin 2009). Given these requirements, any baseline subtraction and threshold setting method can be used with the IPC.

If many different assays are used, amplification curves are rarely all parallel (Figure 6). A single IPC per channel can still be used but different thresholds optimized for each assay may have to be used. The thresholds should be set at a level where the assay and the IPC response curves are parallel (Figure 5, 6).

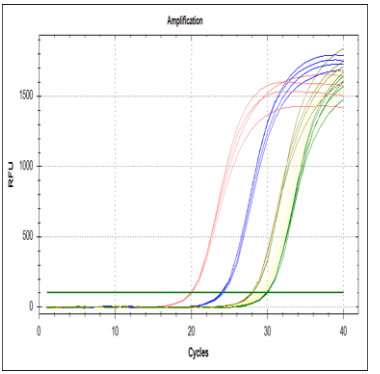


Figure 5: Blue amplification curves are TATAA Interplate Calibrator. The curves are parallel in complete range.

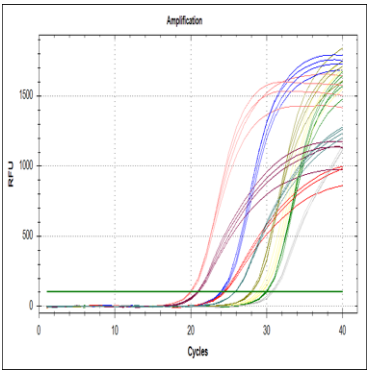


Figure 6: Blue amplification curves are TATAA Interplate Calibrator. The thresholds should be set at a level where the assay and the IPC response curves are parallel.

Different threshold settings:

- **2nd derivative threshold, common threshold (manual or automatic), best fit, SD of noise:**
  TATAA Interplate Calibrator can be used if all the assays are well optimized and show similar amplification curves at least up to threshold

- **Assay dependent threshold:**
  If different thresholds are set for different assays (eg. because of very different probe fluorescence) a single TATAA Interplate Calibrator can still be used for all assays measured in the same instrument channel. For every threshold setting an IPC C_q value is read and used for correction;

- **Inter-instrument calibration:**
  If parts of a qPCR study must be measured on a different instrument (not recommended), but still using the same protocol, the TATAA Interplate Calibrator can be used to remove most of the systematic variation.

Protocol - Interplate calibration

1. Design your experiment and plan where to include the TATAA Interplate Calibrator. We recommend running **minimum of three qPCR technical replicates of the TATAA IPC** in every run.

Recommendation: Putting the IPC in the same position on the plate in every run makes analysis more convenient (uniformity should not be a problem on a well calibrated qPCR instrument).

2. Use in-house PCR reagents and recommended primer concentration for qPCR. Add 2 µl of TATAA Interplate Calibrator template ($2*10^6$ copies) in each qPCR replicate. Expected C_q value is 15 –20.

Recommendation: Prepare for a slightly larger number of reactions to avoid running out of master mix during pipetting. Add all components, vortex gently, spin down and dispense in replicates. The primer stock concentration is 2 µM, note the different concentration compared to other TATAA primer products. This to achieve more accurate liquid handling for such a low number of replicates. We advise adding 2 µl of IPC per sample as pipetting of larger volumes is more accurate. It is not necessary to include a non-template control (NTC) for IPC.

3. Perform qPCR with the protocol recommended for your reagents and as optimized for your assays. For probe-based assays often 60°C annealing temperature is used on two step protocols and qPCR conditions that have been validated for the TATAA Interplate Calibrator are shown in Table 1. The amplicon produced by the TATAA IPC assay is 100 bp long.

4. Inspect data. The amplification curves of the TATAA Interplate Calibrator (IPC) assay should be parallel with those of your assays at least up to threshold (if it is not, see section "additional information"). Collect the C_q values for all the runs and average those of the IPC replicates in each run. The standard deviation (SD) of IPC replicates should be ≤ 0.3 cycles and usually it is substantially lower (the reproducibility is usually limited by the performance of your qPCR instrument).Data are corrected for the variation between runs using the equation:

$$Cq_i^{corrected} = Cq_i^{uncorrected} - Cq_i^{IPC} + \frac{1}{\text{no. plates}} \sum_{i=1}^{\text{no. plates}} Cq_i^{IPC}$$

For each plate subtract the Cq_i^{IPC} from the measured $Cq_i^{uncorrected}$ and add the average of the C_q's off all IPCs ($\frac{1}{\text{no. plates}} \sum_{i=1}^{\text{no. plates}} Cq_i^{IPC}$).

For convenient analysis, interplate calibration is part of the qPCR data analysis workflow in softwares such as GenEx.

GenEx

To enable automatic analysis, runs and interplate calibrators should be indexed in classification columns. It is easy to do this manually, however if you use pre-plated reactions by leading vendors GenEx will automatically identify interplate calibrators and annotate your experiment accordingly. A free license for GenEx Enterprise is available for download from www.multid.se and provides the fully functional analysis software for a trial period of 30 days. To purchase GenEx licenses or for qPCR data analysis services, contact us at order@tataa.com.

GenEx is market-leading software for qPCR experimental design and data processing, and is supported by all leading qPCR instrument manufacturers. It offers user-friendly optimized workflows for qPCR data pre-processing and analysis, including normalization using spikes and identification of inhibited outliers. Pre-processing includes interplate calibration, efficiency correction, various normalization options, handling of technical replicates and missing data, normalization with paired samples, and correction for gDNA contamination using ValidPrime™. Analyses include absolute quantification, relative quantification, and expression profiling. Tutorials are available on: www.multid.se/tutorials.php and free support is offered on: www.qpcrforum.com.

Troubleshooting

• I do not get any amplification/signal?
The instrument may not have been programmed correctly or there may be a problem with the master mix. Establish if the problem is in the detection or the amplification step by running the samples on a gel. Run a new test using the IPC template with the IPC assay provided. If the problem persists, contact us at info@tataa.com.

• My replicates are not tight?
With TATAA Interplate Calibrator template and primers and good pipetting technique, high reproducibility is expected (SD ≤ 0.3 C_q) in all master mixes. SD ≤ 0.5 cycles can still be accepted, but the number of replicates should be increased for accurate interplate calibration. If other assays show such a low reproducibility, it is possible that the qPCR instrument is not performing well and should be validated (test for uniformity of the thermal block). Low amounts of template can lead to higher variation. Also, low quality RNA/DNA can lead to differences between replicates. Check the accuracy and reproducibility of your pipettes.

• My negative controls are amplified?
Your reagents are probably contaminated.

• My samples have same/higher C_q-value than my NTC?
You have used too little template or complete inhibition is present. Add more RNA/DNA and try again. Check if the quality of the RNA/DNA is not compromised due to improper storage before performing RT-qPCR. Check if the instrument is set optimally.

References

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

Kubista M, Rusnakova V, Svec D, Sjögreen B, Tichopad A. GenEx - Data Analysis Software. In qPCR in Applied Microbiology. Editor: Martin Filion. Horizon Press, 2012.

Tichopad A, Svec D, Pfaffl M, Kubista M. How good is a PCR efficiency estimate: Proposal of recommendations for precise and robust qPCR efficiency estimation. Nucleic Acid Research. 2012.

GenEx user guide: http://www.tataa.com/files/pdf/GenExUserGuide.pdf

Reorder information

The TATAA Interplate Calibrator can be ordered from the TATAA webshop on www.tataa.com, by mail: order@tataa.com, or from the TATAA distributor in your country.

Contact

For more information about TATAA Interplate Calibrator, please contact us at info@tataa.com

License information

PCR is covered by several patents owned by Hoffman-La Roche Inc., and Hoffman-LaRoche, Ltd. Purchase of this kit does not include or provide a license with respect to any PCR related patents owned by Hoffman-La Roche or others. TATAA Biocenter does not encourage or support the unauthorised or unlicensed use of the PCR process.

Other products from TATAA

Universal RNA / DNA spike

- for any species, tests for inhibition and yield

The TATAA Universal Spike is an easy to use and very effective tool for quality control throughout entire RT-qPCR experimental workflow. Add the spike to the experimental sample and to a control based on water. Processing both samples exactly the same way – any inhibition in the experimental sample will impair the RT-qPCR resulting in higher C_q than of the control sample. TATAA Universal Spikes have a synthetic sequence that is not present in any known living organism. The Spike assay is exceedingly robust and is optimized for high sensitivity for inhibition. The C_q of the Spike assay also reflects losses during extraction, handling, transport, and storage of samples, including freeze-thaw events during RT-qPCR.

ValidPrime™ - mouse, human and other vertebrates

ValidPrime™ is an assay to test for the presence of gDNA in test samples and when combined with a gDNA control sample, replaces all RT(-) controls. ValidPrime™ is highly optimized and specific to a non-transcribed locus of gDNA that is present in exactly one copy per haploid normal genome. The kit also contains a gDNA standard that can be used to test the sensitivity of RT-qPCR assays for gDNA background. ValidPrime™ replaces the need to perform RT(-) controls for all reactions and makes RT-qPCR profiling easier and substantially cheaper.

HL-dsDNase

New generation DNase from Arcticzymes that is specific to double strand DNA and can be efficiently inactivated by heating at 55°C. It can be added to your RT reaction to efficiently remove any gDNA, without degrading single-stranded cDNA. It is completely inactivated by the PCR and does not degrade the double stranded PCR product.

GenEx software

Market leading software for qPCR analysis from MultiD Analyses. GenEx provides the appropriate tools to analyze qPCR gene expression data and to extract biologically relevant information from the measurements.

Reference Gene Panel - Human, Mouse, or Rat

The panel contains primer sets for 12 commonly used human, mouse, or rat reference genes. A perfect product for finding the most optimal reference genes for your study. A one year license for GenEx Standard software with geNorm and Normfinder is included with the kit.

VisiBlue™ qPCR mix colorant

The VisiBlue™ mastermix colorant enables you to color your favourite qPCR mastermix to easily visualize where the reagent is loaded to your plates and tubes. VisiBlue is very easy to use by simple addition to your favorite master mix.

CelluLyser™ - for rapid and easy lysis and cDNA synthesis

The CelluLyser™ Lysis and cDNA Synthesis Kit enables you to generate cDNA from small samples with minimal losses and hands-on time. It is particularly useful for single cell analysis. By using CelluLyser™, the entire workflow from cell lysis to RT and qPCR can be performed without washing steps, thus eliminating material loss.

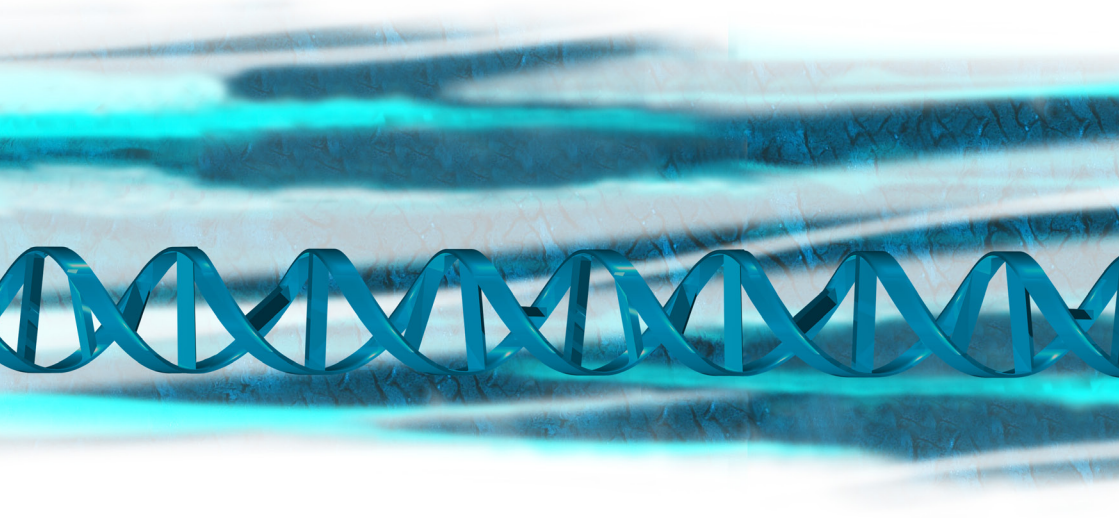
TATAA GrandMaster® and GrandScript Series

After specializing in qPCR for more than a decade TATAA Biocenter now introduces its own series of mixes and cDNA synthesis kits for optimal and high quality results. Our mission is to deliver areagent series that provides superior qPCR performances in a variety of applications and throughout the entire qPCR workflow.

qPCR training courses at TATAA Biocenter

TATAA Biocenter is leading organizer of hands-on training in qPCR and related technologies. For comprehensive training program see www.tataa.com.

Express your genius



TATAA Biocenter, with offices in Gothenburg, San Francisco and Prague is the leading provider of real-time PCR services and the prime organizer of real-time PCR workshops globally. TATAA Biocenter conducts commissioned research and training within the field of molecular diagnostics and gene expression analysis, along with developing real-time PCR expression panels. TATAA Biocenter has great experience and expertise in high resolution gene expression profiling, pathogen detection, and small sample/single cell analysis.

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